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A Cluster of Ribosome Synthesis Factors Regulate Pre-rRNA Folding and 5.8S rRNA Maturation by the Rat1 Exonuclease

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 26 January 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments are shown below. As you will see while referee 1 is more critical the other two referees are more positive and would support publication here after appropriate revision. Still, all three referees express concerns regarding the mainly speculative nature of your model (figure 7) put forward in the discussion section. Referee 1 feels strongly that this model should be tested by a number of further experiments. I would thus like to invite you to prepare a revised manuscript that addresses or responds to the points put forward by referees. I would like to urge you to include at least some more experiments that test your model along the lines put forward by referee 1.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Please do not hesitate to contact me at any time should you wish to discuss issues regarding the revision further.

Thank you for the opportunity to consider your work for publication. I look forward to your

revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

EMBOJ-2010-76863

In this manuscript Granneman and colleagues use the CRAC crosslinking method to map the binding sites for 5 (out of seven) members of a protein cluster required for Rat1/Rai1-dependent rRNA processing from site A3 to BS. In addition the sites of Rat1 crosslinking are mapped both in wild type strains as well as in the absence of Nop7, or Nop15, two members of the so-called A3 cluster. This manuscript provides provocative ideas that are unfortunately not supported by any data. The authors should either take these data and publish them in a more specialized journal (e.g. RNA), or do the experiments to test their very interesting models. Details are below.

Major points:

1. The data are interpreted in terms of a model whereby there is a conformational change in ITS1. In the specific model there are initial base pairs within ITS1 and 5.8S, which are disrupted to form basepairs between 5.8S and 25S. This is based on a 20 year old paper, in which in vitro transcribed 35S rRNA was probed. Part of that paper suggested that cleavage site D was in a duplex, which the authors themselves have now shown is wrong. Furthermore, there is no phylogenetic conservation for the proposed base pairs and C albicans does not even contain the involved nucleotides. If the authors want to include a model in which ITS1 is dynamic, they need to first provide evidence for the in vivo (and in vitro) formation of the suggested base pairs between ITS1 and 5.8S rRNAs.

2. Similarly the authors propose that the A3 cluster proteins are required for proposed changes in ITS2. If that is the case, one would expect in vivo footprinting for ITS2 to differ in the absence and presence of these proteins. This experiment needs to be done.

3. The authors make a big deal about the fact that the crosslinking sites for the A3 cluster proteins do not cluster near the A3 site; we have no idea where the A3 site is located in 3D; it could be over the sites shown; there's ~ 60 nt separating A3 from 5.8S, these can span about any distance

4. Most importantly, the CRAC technique is an equilibrium technique; everything in a mixture can crosslink, more abundant species should give more crosslinks (assuming equal interactions); 27SA3, the substrate for the A3 cluster proteins and Rat1 is NOT abundant; in contrast, both the 27SA2 (upstream) and even more so the 27SB (downstream product) are very abundant. In the simplest model, that would suggest that crosslinking should reflect on the 27SB complex, as well as the 27SA2 complex, and given the lack of 27SA3 in the steady state, this RNA should produce little crosslinks; this readily explains all the data: the crosslinking likely occurs after 27SA3 has been processed to 27SBS; thus, one cannot actually lean much about what needs to happen for processing to occur;

5. This model also explains the changes in Rat1 crosslinking upon depletion of Nop7 or Nop15; previous data has shown that upon deletion of the A3 cluster proteins the 27SA3 intermediate accumulates at the expense of the 27SBS; the simplest interpretation of these data is not that Rat1 recruitment is affected but that the crosslinks at the 3'-end of 5.8S and in ITS2 that are reduced upon Nop7/15 depletion occur in 27SB (the wild type most abundant species); in contrast the crosslinks at the 5'-end of 25S (which is base paired to the 3'-end of 5.8S) increase, similar to the levels of 27SA3, indicating that these might actually occur in the 27SA3 species, the relevant one; this is near

the sites for Nop7 and Erb1; crosslinks e g in 5'-ETS do not change as the abundance of the intermediate in which these crosslinks occur does not change

6. Surprisingly, the Northerns in Fig. S4 do not actually show the phenotype of 27SA3 accumulation for Nop7, even though it has been published twice, including by the Tollervey lab. Furthermore, the phenotype from Nop7 depletion is different from the phenotype observed with Nop15 depletion, which is in line with the previously published Nop7 data. How could this be if depletion of one proteins leads to depletion of all proteins, as stated multiple times, so that Nop7 depletion should be identical to Nop15 depletion. Please clear up this discrepancy with previously published data and between data herein.

7. His-tagging (esp. of Cic1 and Nop15) leads to small perturbations in the levels of rRNAs (Fig.5), indicating that these proteins are not fully functional; this is likely to affect the crosslinking.

8. Why are the sites for the other proteins not included in Figure 4, instead all of these Rpl are shown? In addition, the lesser sites are also not shown, but likely relevant (H10~H66, for Nop12), why omit data selectively?

9. There is no functional evidence linking Rat1 to 5'-ETS degradation other than A0-A1, the authors should either provide such data or not claim it does.

10. One of the most interesting findings is the observation of very high crosslinking in ITS2 BEFORE C2, this either indicates that the technique is prone to artifacts (although there are controls), but more interestingly, these data could indicate that Rat1 is "waiting" there for cleavage at site C2 to occur.

Minor points:

11. the paper is exceedingly poorly put together; it is full of typos, figures that are misreferenced etc., this does not reflect well. Examples:
a. abstract, first sentence: Rat1 degrades spacer fragments, plural;
b. abstract, A3 is sometimes as A3 and sometimes as A3, the conventional denotation. Settle on one way;
c. the introduction references Figure 2C, really the reference should be to Fig. 3C
d. first paragraph of introduction, last sentence: these data are used to interpret the sites identified here. Sites of what? crosslinking has not been mentioned, yet
e. introduction, last paragraph: missing the words "the" and "of" in the second sentence
f. p.11, second paragraph, first sentence has the word "the" twice
12. It is not clear why multiple spots of Rat1 cleavage provide any evidence for dynamic changes in rat1 association, as opposed to many different intermediates and steps that Rat1 is involved in (p.6, top).

Referee #2 (Remarks to the Author):

The process of ribosome assembly in eukaryotes has turned out to be extremely complex. Over the past few years a catalogue of hundreds of proteins involved in this process has been generated. Now the problem is to identify where and when each of these act. This is a very nice paper which attacks that problem. The authors use UV cross-linking to locate a few of the ribosome assembly factors on the nascent ribosome, and are able to draw quite novel and convincing conclusions about the arrangement of several important molecules in relation to the pre-rRNA transcript. The Discussion goes quite a way beyond this, furnishing a host of possibilities that can be experimentally attacked. Only in one case, see 4 below, do I suggest that they have gone too far beyond their data.
Minor points:

1) P 5 There is no Fig 2B. Also the asterisks are not explained, though they are apparently from the negative controls, except for the one over the B0/1 site.

2) P5 mid There actually seems to be LESS x-link at 5' end of 25S than within it. Interpretation?

3) I realize that Fig 3C is complex enough, but indicating the helices H8, etc would be helpful.

4) P12,l22 Site B0 is not indicated on Fig 1A, making it hard for the non-aficianado to understand this argument. Only a minority of molecules, except in the most rapidly growing cells, are cleaved at A2 before B0, so it is unclear that this is a strong argument. In any case, the data in this Ms does not pertain to the kinetics by which Rat1 binds to the transcript.

Referee #3 (Remarks to the Author):

In this manuscript, Dr. Tollervey and co-workers use the CRAC method to detect pre-rRNA interaction positions for a set of trans-acting factors involved in 60S ribosomal subunit biogenesis: Rat1, which is an 5'-3' exonuclease required for processing from A3 to B1S site and from the C2 to C1 site and Nop7, Erb1, Nsa3/Cic1, Nop15 and Nop12, which are all factors from the so-called "A3-cluster" that are required for processing from A3 to B1S site and pre-rRNA processing at ITS2. Moreover, they study the Rat1-binding sites across the pre-rRNA upon depletion of two members of the "A3-cluster", Nop7 and Nop15. From all data obtained, they conclude that Rat1 does not bind near the A3 site that it attacks but mainly binds regions of 5.8S/25S rRNAs (helix H3 and H4) and ITS2 spacer. The binding to the ITS2 spacer is dependent on the presence of at least Nop7 and Nop15 proteins. All together, the data allow the authors to establish a very speculative model to explain how a rearrangement in ITS2 would allow Rat1 to exonucleolytically act on the A3 site. In this rearrangement, both a set of 60S ribosomal proteins (Rpl17, Rpl25, Rpl26, Rpl35) and the A3-cluster factors play an essential role.

CRAC analysis, which was developed by Dr. Granemman at the Tollervey's lab, is a very novel and useful method to map in vivo protein-RNA interactions at the level of nucleotide. This is the fourth paper of this lab on this technique and in all cases the analysis allow a better understanding of the molecular function of the factors strategically selected.

The work is nice and of high quality. I only find minor critique that would be addressed to match the scopes of the prestigious EMBO Journal.

(a) Summary: Rat1 is involved in 5' maturation of only 5.8Ss rRNA. The third sentence ("the 5.8S 5' end is ...") should be modified consequently.

(b) Introduction: change citation of Figure 2C to Figure 3C.

(c) Introduction: Sentence "However, secondary structure ... conformational switch" requires a reference, for instance those from Peculis' lab.

(d) Introduction: Sentence "The mechanism of formation of the 5' end of the less abundant 5.8SL... " is not totally accurate. The work of Faber et al. BBRC 345: 796-802, 2006 demonstrated that 5' end formation of 5.8SL rRNA is an endonucleolytic event.

(e) Introduction: Authors mentioned that loss of factors from the A3-cluster group (including Rlp7, Nop15, Nsa3/Cic1, Rrp1, Nop7, Erb1, Ytm1) and Nop12 leads to "accumulation of 27SA3 pre-rRNA and reduced synthesis of 27SBs and mature 5.8Ss rRNA, without concomintant loss of 27SBL or 5.8SL". I have checked the literature and this statement is not completely clear to me. Due to the fact that one of the take-home messages of this article is that Rat1 activity at the A3 site is dependent on the A3-cluster proteins, I strongly recommend the authors to analyse the effects of depletion or mutation of any of the abovementioned factors on pre-rRNA processing by both primer extension and northern blotting. All factors studied in this work (Rat1, Nop12, Nsa3/Cic1, Nop15, Nop7, Erb1) would be analysed in the same figure. Some of this request is performed in Figure S4B and S4C but I consider it still incomplete.

(f) Figure 1: I find two possible inconsistencies with this figure.

- Tollervey's lab has nicely demonstrated that the final steps in 5.8S rRNA maturation (6S to 5.8S) occurs in the cytoplasm (Thomson and Tollervey, MCB 2010) but this information is not represented as such in the current figure.

- In Figure 1A, some trans-acting factors are indicated but not all were positive for the CRAC analysis of this work (i.e. Brx1, Pwp1, Ytm1). Moreover, the dissociation time point is not depicted for all factors.

(g) Negative control for CRAC. Please specify what band of interest is cut out to perform the analysis (see Figure S1B). In theory, in such negative control there is no band to cut. Is this correct? Please explain in material and methods.

(h) I would include an independent Figure similar to Figure 3B with all pre-rRNA interaction sites for Rat1 in the pre-rRNA.

(i) In my opinion, Figure 5 clearly needs quantification to assess the percentage of each RNA recovered after purification of the selected factors.

(j) Figure 6: Loss of Nop7 or Nop15 also alter the distribution of Rat1 cross-linking to the A2-A3 region, however, this is neither comment in the result nor in the discussion section.

(k) Model of Figure 7 is, as the author described, speculative. I imagine that testing experimentally this model would not be an obvious task. I demand the authors to at least propose in the discussion section possible approaches that could be followed to test it (i.e. chemical probing on RNA purified from homogeneous pre-ribosomal particles up and downstream the main rearrangement of ITS2 ?).

(l) Authors described the CRAC medium, but why do they use a medium with galactose and raffinose for the strain harbouring the HTP-tagged Rat1 under its own promoter?

(m) Figure S3. I guess the red box for Nop15 is missing. Is this correct?

(n) Figure S4. See comment e. Moreover, labelling 5.8SS+L is not correct since this primer extension stop corresponds to the B1S+L site of 7S and 27SB pre-rRNAs if I correctly interpreted where primer 007 hybridises.

1st Revision - authors' response 28 April 2011

Referee #1

Major points:

1. The data are interpreted in terms of a model whereby there is a conformational change in ITS1. In the specific model there are initial base pairs within ITS1 and 5.8S, which are disrupted to form basepairs between 5.8S and 25S. This is based on a 20 year old paper, in which in vitro transcribed 35S rRNA was probed. Part of that paper suggested that cleavage site D was in a duplex, which the authors themselves have now shown is wrong. Furthermore, there is no phylogenetic conservation for the proposed base pairs and C albicans does not even contain the involved nucleotides. If the authors want to include a model in which ITS1 is dynamic, they need to first provide evidence for the in vivo (and in vitro) formation of the suggested base pairs between ITS1 and 5.8S rRNAs.

We now present *in vivo* and *in vitro* DMS modification experiments (Figure 7 and Supplementary Figure S6), which are consistent with the model proposed by Yeh et al.

2. Similarly the authors propose that the A3 cluster proteins are required for proposed changes in ITS2. If that is the case, one would expect in vivo footprinting for ITS2 to differ in the absence and presence of these proteins. This experiment needs to be done.

This has also been addressed by structure probing in the revised MS.

3. The authors make a big deal about the fact that the crosslinking sites for the A3 cluster proteins do not cluster near the A3 site; we have no idea where the A3 site is located in 3D; it could be over the sites shown; there's ~ 60 nt separating A3 from 5.8S, these can span about any distance

We had anticipated that at least some of the proteins would bind to the pre-rRNA in proximity to the site at which their effects are manifested. As the referee points out - we lack clear structural information on the pre-ribosomes, and this is what we are trying to address.

4. Most importantly, the CRAC technique is an equilibrium technique; everything in a mixture can crosslink, more abundant species should give more crosslinks (assuming equal interactions); 27SA3, the substrate for the A3 cluster proteins and Rat1 is NOT abundant; in contrast, both the 27SA2 (upstream) and even more so the 27SB (downstream product) are very abundant. In the simplest model, that would suggest that crosslinking should reflect on the 27SB complex, as well as the 27SA2 complex, and given the lack of 27SA3 in the steady state, this RNA should produce little crosslinks; this readily explains all the data: the crosslinking likely occurs after 27SA3 has been processed to 27SBS; thus, one cannot actually lean much about what needs to happen for processing to occur;

5. This model also explains the changes in Rat1 crosslinking upon depletion of Nop7 or Nop15; previous data has shown that upon deletion of the A3 cluster proteins the 27SA3 intermediate accumulates at the expense of the 27SBS; the simplest interpretation of these data is not that Rat1 recruitment is affected but that the crosslinks at the 3'-end of 5.8S and in ITS2 that are reduced upon Nop7/15 depletion occur in 27SB (the wild type most abundant species); in contrast the crosslinks at the 5'-end of 25S (which is base paired to the 3'-end of 5.8S) increase, similar to the levels of 27SA3, indicating that these might actually occur in the 27SA3 species, the relevant one; this is near the sites for Nop7 and Erb1; crosslinks e g in 5'-ETS do not change as the abundance of the intermediate in which these crosslinks occur does not change

We have reinterpreted several of these results in the light of the analysis of Nop4 (see below), which is required for pre-rRNA cleavage at sites A3 and B1L. The loss of Rat1 association in Nop4 and Nop15 depleted cells is similar, showing that the effects are not specific for the A3 cluster proteins.

6. Surprisingly, the Northerns in Fig. S4 do not actually show the phenotype of 27SA3 accumulation for Nop7, even though it has been published twice, including by the Tollervey lab. Furthermore, the phenotype from Nop7 depletion is different from the phenotype observed with Nop15 depletion, which is in line with the previously published Nop7 data. How could this be if depletion of one proteins leads to depletion of all proteins, as stated multiple times, so that Nop7 depletion should be identical to Nop15 depletion. Please clear up this discrepancy with previously published data and between data herein.

The referee was entirely correct and we are grateful that their diligence has avoided a most unfortunate error. We reanalyzed the strain and found a problem with the integrated construct, which was in fact P_{GAL}-NOP4. In Fig. S4, this panel has been replaced with data from a confirmed Nop7 depleted strain. We have now included side-by-side comparisons of all depletion strains, each of which was verified. This construct was also used in combination with the Rat1-HTP, so these results have been reinterpreted in light of the finding that Nop4 had actually been depleted.

7. His-tagging (esp. of Cic1 and Nop15) leads to small perturbations in the levels of rRNAs (Fig.5), indicating that these proteins are not fully functional; this is likely to affect the crosslinking.

The criticism that tagging may impair protein function can (with some justification) be leveled at almost any analysis that makes use of tagged proteins. Despite this, tagged proteins have been informative in many analyses.

8. Why are the sites for the other proteins not included in Figure 4, instead all of these Rpl are shown? In addition, the lesser sites are also not shown, but likely relevant (H10~H66, for Nop12), why omit data selectively?

The R-protein binding sites are shown because they are directly relevant to our interpretation of the

data. We have now included the Nop12 binding sites in H10 in the revised Figure 4. H66 showed up as a strong peak in several negative control experiments and was therefore not considered a *bona fide* Nop12 binding site. We tried to include other binding sites, however, we realized that we could not show all sites and retain a comprehensible figure.

9. There is no functional evidence linking Rat1 to 5'-ETS degradation other than A0-A1, the authors should either provide such data or not claim it does.

There is strong evidence for endonuclease cleavage at sites in the 5' region of the ETS. The nuclear TRAMP and exosome complexes are clearly responsible for 3' degradation showing that degradation is nuclear. It is a formal possibly that 5' degradation is entirely dependent on Rrp17, the only other known nuclear 5' exonuclease, but this really does not seem very likely. Moreover, the human homologue of Rat1, Xrn2, has been shown to have this function. We have altered the text to reflect the potential uncertainty.

10. One of the most interesting findings is the observation of very high crosslinking in ITS2 BEFORE C2, this either indicates that the technique is prone to artifacts (although there are controls), but more interestingly, these data could indicate that Rat1 is "waiting" there for cleavage at site C2 to occur.

This is also our interpretation and we have altered the revised text to make this clearer.

Minor points:

11. the paper is exceedingly poorly put together; it is full of typos, figures that are misreferenced etc., this does not reflect well. Examples:
a. abstract, first sentence: Rat1 degrades spacer fragments, plural;
b. abstract, A3 is sometimes as A3 and sometimes as A3, the conventional denotation. Settle on one way;
c. the introduction references Figure 2C, really the reference should be to Fig. 3C
d. first paragraph of introduction, last sentence: these data are used to interpret the sites identified here. Sites of what? crosslinking has not been mentioned, yet
e. introduction, last paragraph: missing the words "the" and "of" in the second sentence
f. p.11, second paragraph, first sentence has the word "the" twice

Typos have been corrected.

12. It is not clear why multiple spots of Rat1 cleavage provide any evidence for dynamic changes in rat1 association, as opposed to many different intermediates and steps that Rat1 is involved in (p.6, top).

Two different points were being discussed in the text: Rat1 binds at many distinct sites, presumably reflecting its multiple target regions. In addition to this, the pattern of dispersed Rat1 crosslinking over the 5.8S region looks quite different from other ribosome synthesis factors that we have analyzed - and it is this pattern that makes us think the interaction is dynamic. We have, however, removed this sentence from the revised text.

Referee #2

The process of ribosome assembly in eukaryotes has turned out to be extremely complex. Over the past few years a catalogue of hundreds of proteins involved in this process has been generated. Now the problem is to identify where and when each of these act. This is a very nice paper which attacks that problem. The authors use UV cross-linking to locate a few of the ribosome assembly factors on the nascent ribosome, and are able to draw quite novel and convincing conclusions about the arrangement of several important molecules in relation to the pre-rRNA transcript. The Discussion goes quite a way beyond this, furnishing a host of possibilities that can be experimentally attacked. Only in one case, see 4 below, do I suggest that they have gone too far beyond their data.
Minor points:

1) P 5 There is no Fig 2B. Also the asterisks are not explained, though they are apparently from the negative controls, except for the one over the B0/1 site.

This has now been corrected. Asterisks highlight presumed contaminant peaks. This is now explained in the figure legend and main text

2) P5 mid There actually seems to be LESS x-link at 5' end of 25S than within it. Interpretation?

We predict that folding of the pre-25S rRNA brings these sites into proximity with ITS2.

3) I realize that Fig 3C is complex enough, but indicating the helices H8, etc would be helpful.

All the helices are now indicated with 'H'

4) P12,l22 Site B0 is not indicated on Fig 1A, making it hard for the non-aficianado to understand this argument. Only a minority of molecules, except in the most rapidly growing cells, are cleaved at A2 before B0, so it is unclear that this is a strong argument. In any case, the data in this Ms does not pertain to the kinetics by which Rat1 binds to the transcript.

B0 has been added to the figure. Metabolic labeling data and EM analyses both indicate that around 70% of pre-rRNA molecules are cleaved at A2 prior to B0. The labeling analyses were performed in medium very similar to that used for the analyses reported here.

Referee #3

(a) Summary: Rat1 is involved in 5' maturation of only 5.8Ss rRNA. The third sentence ("the 5.8S 5' end is ...") should be modified consequently.

Corrected

(b) Introduction: change citation of Figure 2C to Figure 3C.

Corrected

(c) Introduction: Sentence "However, secondary structure ... conformational switch" requires a reference, for instance those from Peculis' lab.

The text refers to a predicted switch in ITS1, which would be distinct from the work of the Peculis lab. We have included the reference to Yeh et al who first reported this structure.

(d) Introduction: Sentence "The mechanism of formation of the 5' end of the less abundant 5.8SL... " is not totally accurate. The work of Faber et al. BBRC 345: 796-802, 2006 demonstrated that 5' end formation of 5.8SL rRNA is an endonucleolytic event.

We have altered the text.

(e) Introduction: Authors mentioned that loss of factors from the A3-cluster group (including Rlp7, Nop15, Nsa3/Cic1, Rrp1, Nop7, Erb1, Ytm1) and Nop12 leads to "accumulation of 27SA3 pre-rRNA and reduced synthesis of 27SBs and mature 5.8Ss rRNA, without concomintant loss of 27SBL or 5.8SL". I have checked the literature and this statement is not completely clear to me. Due to the fact that one of the take-home messages of this article is that Rat1 activity at the A3 site is dependent on the A3-cluster proteins, I strongly recommend the authors to analyse the effects of depletion or mutation of any of the abovementioned factors on pre-rRNA processing by both primer extension and northern blotting. All factors studied in this work (Rat1, Nop12, Nsa3/Cic1, Nop15, Nop7, Erb1) would be analysed in the same figure. Some of this request is performed in Figure S4B and S4C but I consider it still incomplete.

The requested analyses have been performed and the data are now shown in the revised Supplementary Figure S4

(f) Figure 1: I find two possible inconsistencies with this figure.

- Tollervey's lab has nicely demonstrated that the final steps in 5.8S rRNA maturation (6S to 5.8S) occurs in the cytoplasm (Thomson and Tollervey, MCB 2010) but this information is not represented as such in the current figure.

We had omitted this step for simplicity as it was not relevant to the current work. It has now been included.

- In Figure 1A, some trans-acting factors are indicated but not all were positive for the CRAC analysis of this work (i.e. Brx1, Pwp1, Ytm1). Moreover, the dissociation time point is not depicted for all factors.

We now show only the relevant factors, together with our best estimates of their dissociation points.

(g) Negative control for CRAC. Please specify what band of interest is cut out to perform the analysis (see Figure S1B). In theory, in such negative control there is no band to cut. Is this correct? Please explain in material and methods.

We excised several different membrane fragments, in the range of ~30-100 kDa, from parallel mock precipitates. Even though there is no discrete band, some lane background is nearly always visible. This information is now included in the Materials and Methods

(h) I would include an independent Figure similar to Figure 3B with all pre-rRNA interaction sites for Rat1 in the pre-rRNA.

Drawing the numerous Rat1 binding sites on the rRNA secondary structure models would create a very busy picture, which would not be very informative. We have, however, now included a multiple sequence alignment as Supplementary Table 2 of sequences extracted from Rat1 CRAC experiments. This should give the reader a better and more complete picture of Rat1 binding sites on the pre-rRNA.

(i) In my opinion, Figure 5 clearly needs quantification to assess the percentage of each RNA recovered after purification of the selected factors.

Quantification has now been performed and the data are included in the revised MS in Supplementary Figure S5.

(j) Figure 6: Loss of Nop7 or Nop15 also alter the distribution of Rat1 cross-linking to the A2-A3 region, however, this is neither comment in the result nor in the discussion section.

The analysis of the effects of depletion of Nop4 greatly aided the interpretation of this observation. This is discussed in the revised MS.

(k) Model of Figure 7 is, as the author described, speculative. I imagine that testing experimentally this model would not be an obvious task. I demand the authors to at least propose in the discussion section possible approaches that could be followed to test it (i.e. chemical probing on RNA purified from homogeneous pre-ribosomal particles up and downstream the main rearrangement of ITS2 ?).

To support the proposed model, we now include in vivo structure probing to confirm the proposed rearrangement in ITS2 (Figs.7 and S6). Since additional material has been included in response to the comments of the referees, we are very short of space in the revised text. We have therefore included only a brief mention of possible future work. Including further details would entail the removal of other sections from the MS.

(l) Authors described the CRAC medium, but why do they use a medium with galactose and raffinose for the strain harbouring the HTP-tagged Rat1 under its own promoter?

We apologize for the confusion. We did not explain this correctly in the Materials and Methods section. We only used the galactose/raffinose medium for the Rat1 CRAC experiments shown in Figures 6 and S6 where analyzed Rat1 cross-linking in the parental and Nop4 or Nop15 depleted strains. For the Rat1 CRAC experiment shown in Figure 2 we used YPD medium. This has now been more clearly explained in the Materials and Methods section and Figure legends.

(m) Figure S3. I guess the red box for Nop15 is missing. Is this correct?

This was a typing error. We could only consistently detect deletions and mutations in the Cic1 CRAC experiments.

(n) Figure S4. See comment e. Moreover, labelling 5.8SS+L is not correct since this primer extension stop corresponds to the B1S+L site of 7S and 27SB pre-rRNAs if I correctly interpreted where primer 007 hybridises.

The reviewer is correct. 007 hybridizes downstream of the C_2 cleavage site and therefore the primer extension stops indicate 27SA/B pre-rRNAs. The figure has been amended.

2nd Editorial Decision 25 May 2011

Thank you for sending us your revised manuscript. In the meantime, referees 1 and 3 have seen it again. As you will see, while referee 3 is more positive, referee 1 still raises major concerns that require further attention. In the meantime, I have also had a chance to run the points still raised by referee 1 by referee 3. He/she agrees with the concerns and thinks at least the revised points 1, 2 and 4 would need to be addressed prior to acceptance. Given the overall positive assessment by referee 3 and originally by referee 2, I would be prepared to allow for an additional round of revision. However, referee 1's concerns need to be addressed in a satisfactory way; and we will need to involve referees 1 and 3 again to evaluate the re-revised manuscript.

I am looking forward to your re-revised manuscript in due course.

Yours sincerely,

Editor
The EMBO Journal

REFFEREE COMMENTS

Referee #1 (Remarks to the Author):

EMBOJ-2010-76863R

In this manuscript Granneman and colleagues use the CRAC crosslinking method to map the binding sites for 5 (out of seven) members of a protein cluster required for Rat1/Rai1-dependent rRNA processing from site A3 to BS. In addition the sites of Rat1 crosslinking are mapped both in wild type strains as well as in the absence of Nop4, or Nop15, two members of the so-called A3 cluster. The authors now also provide in vivo and In vitro DMS modification data. While the revised manuscript addresses many of the issues brought up in the previous review, there are still many remaining issues, which must be addressed prior to publication.

Major points:

1. Original point: The data are interpreted in terms of a model whereby there is a conformational change in ITS1. In the specific model there are initial base pairs within ITS1 and 5.8S, which are disrupted to form base pairs between 5.8S and 25S. This is based on a 20 year old paper, in which in vitro transcribed 35S rRNA was probed. Part of that paper suggested that cleavage site D was in a

duplex, which the authors themselves have now shown is wrong. Furthermore, there is no phylogenetic conservation for the proposed base pairs and C albicans does not even contain the involved nucleotides. If the authors want to include a model in which ITS1 is dynamic, they need to first provide evidence for the in vivo (and in vitro) formation of the suggested base pairs between ITS1 and 5.8S rRNAs.

Revised point: While the authors have carried out the suggested DMS modification experiment (Fig. S7), and are highly commended for this, the analysis of these data is seriously lacking. First of all, the gel is overexposed, which precluded the analysis of many sites of modification, simply the use of photoshop to reduce the exposure could help. The sites of modification in the gel could be labeled with a number, so that it is easier to tell which position on the gel corresponds to which on the secondary structure map. Most of all, the data are interpreted in terms of supporting one secondary structure model, versus another (the secondary structure in Fig. S7 versus the one in Figure 3). Minimally, both secondary structures should be shown in this figure and the modifications mapped. I believe that this analysis will indeed show that the obtained modification patterns is NOT really consistent with the proposed structure by Yeh. Specifically, only certain As are modified but not neighboring ones, which should be according to the Yeh model. Also, I am somewhat concerned about the data, as the primer extension in Fig. S4, as well as Northern data all suggest that in the Cic1 strains no cleavage at site B1s occurs, as 27SA3 is accumulated. Due to the overexposure it is really hard to tell, but it does seem that there is no reduction in the strong DMS independent stop at B1S in the Cic1 strain, which suggests that perhaps the authors are not using RNA from Cic1 depleted strains.

Furthermore, the lack of conservation has not been addressed by the authors. The rRNA sequences for hundreds of organisms are in databases and have recently been analyzed for a similar study in which a secondary structure switch was demonstrated (Lamanna&Karbstein, JMB 2011). The analysis of rRNA cleavage in dictyostelium was also recently published (Boesler et al., JBC 2011).

2. Original point: Similarly the authors propose that the A3 cluster proteins are required for proposed changes in ITS2. If that is the case, one would expect in vivo footprinting for ITS2 to differ in the absence and presence of these proteins. This experiment needs to be done.

Revised point: The authors have also carried out this experiment (Figure 7), and produce data of extraordinary quality, but this experiment has possibly even more flaws than the previous one, as described below.

• It does not seem that the authors know that in the interpretation of the DMS gels, one needs to go up 1 nucleotide in the sequencing lane, as the RT falls of at the modification, but the sequencing stops 1 nucleotide after the ddNTP incorporation). To my eye it seems like most residues are misassigned by one nucleotide (it is very hard to tell for sure though because the figure is so small, perhaps showing a full page figure in the supplemental data would help; this comment also applies to Fig. S7). Minimally, the authors must label the entire gel better. Residues that they want to discuss should be labeled with the residue number, e.g. C2 and C5, on the gel. This would help navigate the gel and the corresponding secondary structure map in Figures 7C and D.

• Considering that every residue is shifted one nucleotide it also seems like many of the most obvious changes are actually Us (the most prominent stops in stem 2 and 3 are uridines). DMS modifies A and C, as well as G. However, to detect G modification one needs to carry out aniline cleavage, which was not done. "U"s cannot be modified by DMS unless there is a tautomeric shift, which requires a pKa change. Thus, "U"s should not be interpreted. This will remove many of the interesting nucleotides from the authors' analysis. Had they labeled the gel better with residue names and numbers, this would have been immediately obvious.

• Furthermore, labeling on the secondary structure does not correspond to what I can see in the gel. Minimally, there is no data shown for the modifications labeled as such in stem VI (Fig. 7C,D), the number of strong stops on the secondary structure does not even correspond to the number of strong stops in the wild type strain, some modifications are not mapped etc.. The secondary structure is also messed up in panel D at stems 1 and 2. This reflects sloppiness of the authors, the same kind of sloppiness that led to the mix-up of strains before.

• The data from the depletion strains fail to support the CRAC data. For example, the analyzed sites overlap with the suggested binding site for Cic1 and Nop15, based on CRAC analysis. However, depleting these proteins does not produce enhanced modification at that site, which would be a very strong prediction from direct protein binding. The same has been previously found for Nob1, where in vivo DMS showed protections different from those observed by CRAC. While the statistics of this are certainly weak (only three analyzed strains), they do suggests that CRAC does NOT report on direct binding as asserted by the authors, but likely on nearby binding, consistent with findings on Prp43 crosslinking by the authors. While I do not believe that this has any serious impact on the usefulness of the technique or even the interpretations herein, I do think that this is an important point, which must be discussed, as it is currently believed in the community to do so. I would even encourage the authors to try to figure out what the radius is for CRAC modification, or if there are any constrains (i.e. only modifications in the minor groove, as found for Fe-EDTA mapping), to help future interpretations of the technique. I must stress though that such an analysis would go clearly beyond the current manuscript and is only for the authors' future considerations.

• Finally, the experiment was carried out to provide evidence for conformational switches promoted by the A3 cluster proteins. There are exactly 2 nucleotides that differ in these strains, C2 and C5. While the changes in their modification are consistent with the model of the ring and hairpin switch and a role of Cic1 and Nop15 in either promoting this switch, none of the other predicted changes can be seen; thus, the data simply seem to suggest that Cic1 and Nop15 prevent the formation of a longer stem II.

• I am also not sure that I would have done the experiment this way. As pointed out by the authors, the DMS reports on all 27S RNAs, in the wild type strain that is mostly 27Sb, and some 27SA2, hardly any 27SA3, which the authors want to analyze; it seems the right strain for analyzing 27SA3 would be one deficient for A2 cleavage, so that 27SA3 accumulates, perhaps even combined with mutations in Rat1 to stall processing after A3. This strain then I would have analyzed +/- Nop15, Cic1 etc.

3. Original point: Surprisingly, the Northerns in Fig. S4 do not actually show the phenotype of 27SA3 accumulation for Nop7, even though it has been published twice, including by the Tollervey lab. Furthermore, the phenotype from Nop7 depletion is different from the phenotype observed with Nop15 depletion, which is in line with the previously published Nop7 data. How could this be if depletion of one proteins leads to depletion of all proteins, as stated multiple times, so that Nop7 depletion should be identical to Nop15 depletion. Please clear up this discrepancy with previously published data and between data herein.

Revised point: This point has been sufficiently addressed. However, I noticed that other experiments previously labeled as Nop7 have been relabeled as Nop4. Has that been checked in every instance, or was it simply assumed that the mix-up happened every time.

4. Original point: This model also explains the changes in Rat1 crosslinking upon depletion of Nop7 or Nop15; previous data has shown that upon deletion of the A3 cluster proteins the 27SA3 intermediate accumulates at the expense of the 27SBS; the simplest interpretation of these data is not that Rat1 recruitment is affected but that the crosslinks at the 3'-end of 5.8S and in ITS2 that are reduced upon Nop7/15 depletion occur in 27SB (the wild type most abundant species); in contrast the crosslinks at the 5'-end of 25S (which is base paired to the 3'-end of 5.8S) increase, similar to the levels of 27SA3, indicating that these might actually occur in the 27SA3 species, the relevant one; this is near the sites for Nop7 and Erb1; crosslinks e g in 5'-ETS do not change as the abundance of the intermediate in which these crosslinks occur does not change

Revised point: This point has been partly addressed. However, I do not understand the author's logic that Nop4 inhibits processing prior to A3 cleavage. That would mean A2 cleavage, and would suggest an effect on 18S production, which is NOT observed. Furthermore, there is not a single reported strain, in which A3 cleavage does not occur but the ratio of 27Sb/27Sl is unchanged. In fact that is a hallmark of missing A3 cleavage, observed in the Rnase MRP mutants. Finally, the Northern analysis demonstrates that NO 27S RNA can be found. This suggests that in the absence of Nop4 27S pre-rRNA is rapidly degraded, consistent with the author's proposal that Nop4 helps bring together domains II and III of 25S rRNA. I suggest that Nop4 stalls after A3 cleavage, hence no effect on the ratio of 27Sl/SB, but that the accumulated intermediate (27SA3, or 27SB) is rapidly

degraded. The remaining RNA is wild type, consistent with the observation that there are no differences between the RNA from this strain and the wild type strain by primer extension/ DMS.

If the authors want to make a point that Rat1 crosslinking depends on a specific protein, they need to look at the same RNA species +/- the desired protein, i.e. Rat1 mutants that accumulate 27SA3 in the presence and absence of their favorite protein. If they don't want to include this experiment, which requires strain building and new experiments and is difficult, they should not draw this conclusion, but say that in model a) Rat1 recruitment depends on these proteins, or that b) it simply reflects the different abundances...and that further experiments must be done to differentiate between these two.

5. Why are the sites for the other proteins not included in Figure 4, instead all of these Rpl are shown? In addition, the lesser sites are also not shown, but likely relevant (H10~H66, for Nop12), why omit data selectively?

Revised point: H8 for Nop12 is still not shown. That data is also intriguing, as it shows crosslinking to only one side of the presumed duplex between 5.8S and 25S rRNA. While one could say it binds to one site of the duplex, the labeled sites of interaction are over more than one turn of the duplex, which means both strands must contact that one side, making these results surprising.

I feel that the data herein map the binding sites for the A3 cluster proteins near 5.8S rRNA, and near the binding sites for the RpL depleted in the absence of the A3 cluster proteins (is that reference submitted from the Tollervey lab?, if so, it should be included in this manuscript). They also show that CRAC crosslinking does NOT report on direct sites of interaction, as previously suggested. Furthermore, they provide data localizing Rat1 near its processing sites. Depletion of Cic1 and Nop15 and in vivo DMS probing indicates that binding of these proteins could prevent formation of a longer stem II in ITS2, but provides no convincing evidence for a role of these proteins in the proposed switch between the hairpin and ring models. The conclusion that there's a switch in ITS1 awaits further analysis (Fig.S7). The interpretation that Rat1 binding requires A3 cluster proteins is also remains unsubstantiated.

If the authors were to substantially revise the manuscript to reflect these pared down conclusions, I would think it is appropriate for publication, although in my opinion it is not clear that that should be in EMBO, as functional data are entirely lacking. I will however leave that for the editor's judgment.

Referee #3 (Remarks to the Author):

This reviewer thinks that the revised version of this manuscript has significantly gained quality regarding the original one.

The authors have practically satisfactorily answered to this reviewer's comments. Most points have adequately been explained. Moreover, most of the additional experiments I suggested have been done and they are convincing. I also believe that points raised by the other referees have properly been addressed.

I still find some critique although minor; I encourage the authors to make the effort to address it:

Point (a) has not been corrected yet, thus sentence starting by "The 5.8S 5' end is generated by Rat1" has not been modified yet to "5.8Ss".

Point (e). Figure S4 is still not totally satisfactory. Authors nicely show accumulation of 27SA3 upon depletion of NSA3, ERB1, NOP7, NOP15 and deletion of NOP12. Unfortunately, the primer extension shown in Fig S4C has no enough resolution to distinguish between 27SBL and 27SBs pre-rRNAs.

Point (h). The authors claimed that drawing the numerous Rat1 binding sites would create a very busy figure which would not be very informative, but honestly, Table S2 provides a never ending

list of raw data (even after Novoalign cooking !) of very difficult digestion.
I wonder if a Figure on the pre-rRNA showing only that Rat1 CRAC related sequences where frequently mutations were found (i.e. boxes as in the case of Figure S3) could be more useful.

Two additional typos:
Figure 5B. Please label first panel on the left with 27SA/B (I guess).
Figure 5C is (similarly to previous point e) still no perfect since levels of 27SBL and 27SBs could not be interpreted from such an overexposed film.
Legend to Figure S4. Please correct sentence "scan shown in Figure S6" to "S7".

2nd Revision - authors' response 10 June 2011

Referee #1 (Remarks to the Author):

EMBOJ-2010-76863R

In this manuscript Granneman and colleagues use the CRAC crosslinking method to map the binding sites for 5 (out of seven) members of a protein cluster required for Rat1/Rai1-dependent rRNA processing from site A3 to BS. In addition the sites of Rat1 crosslinking are mapped both in wild type strains as well as in the absence of Nop4, or Nop15, two members of the so-called A3 cluster. The authors now also provide in vivo and In vitro DMS modification data. While the revised manuscript addresses many of the issues brought up in the previous review, there are still many remaining issues, which must be addressed prior to publication.

Major points:

1. Original point: The data are interpreted in terms of a model whereby there is a conformational change in ITS1. In the specific model there are initial base pairs within ITS1 and 5.8S, which are disrupted to form base pairs between 5.8S and 25S. This is based on a 20 year old paper, in which in vitro transcribed 35S rRNA was probed. Part of that paper suggested that cleavage site D was in a duplex, which the authors themselves have now shown is wrong. Furthermore, there is no phylogenetic conservation for the proposed base pairs and C albicans does not even contain the involved nucleotides. If the authors want to include a model in which ITS1 is dynamic, they need to first provide evidence for the in vivo (and in vitro) formation of the suggested base pairs between ITS1 and 5.8S rRNAs.

Revised point: While the authors have carried out the suggested DMS modification experiment (Fig. S7), and are highly commended for this, the analysis of these data is seriously lacking. First of all, the gel is overexposed, which precluded the analysis of many sites of modification, simply the use of photoshop to reduce the exposure could help. The sites of modification in the gel could be labeled with a number, so that it is easier to tell which position on the gel corresponds to which on the secondary structure map. Most of all, the data are interpreted in terms of supporting one secondary structure model, versus another (the secondary structure in Fig. S7 versus the one in Figure 3). Minimally, both secondary structures should be shown in this figure and the modifications mapped. I believe that this analysis will indeed show that the obtained modification patterns is NOT really consistent with the proposed structure by Yeh. Specifically, only certain As are modified but not neighboring ones, which should be according to the Yeh model.

We are unclear why the referee attaches such importance to this point, but feel that a definitive analysis of the ITS1 pre-rRNA structure is both tangential to the topic and beyond the scope of the present work. Our structure probing is in agreement with previously published structure probing data and with published phylogenetic data, and clearly supports the existence of a stem structure in this region. The presence of this stem is also consistent with the fragments that are accumulated in published exonuclease mutant analyses. As requested we have added a cartoon of the 5.8S-25S base paired interaction to Fig. S7. The modification in the 5.8S 5' region is not compatible with the mature structure, although the number of reactive nucleotides is low. The structure probing is performed in vivo on RNA-protein complexes, and it therefore seems unsurprising that not all unpaired nucleotides are reactive. As the referee notes, Lamanna & Karbstein (JMB 2011) have

recently reported a structural analysis of the ITS1 region and we feel that a more extensive analysis is beyond the scope of the present work.

Furthermore, the lack of conservation has not been addressed by the authors. The rRNA sequences for hundreds of organisms are in databases and have recently been analyzed for a similar study in which a secondary structure switch was demonstrated (Lamanna&Karbstein, JMB 2011). The analysis of rRNA cleavage in dictyostelium was also recently published (Boesler et al., JBC 2011).

A phylogenetic analysis of ITS1, including the ITS1-5.8S proximal region has been published and was used as the basis of functional analyses (van Nues et al. (1994,) Nucleic Acids Res. 22, 912-919). The results were explicitly stated by the authors to be in agreement with the structure of Yeh et al. We have altered the text in the revised Discussion to emphasize this point. It is unclear what will be gained by repeating these analyses.

Also, I am somewhat concerned about the data, as the primer extension in Fig. S4, as well as Northern data all suggest that in the Cic1 strains no cleavage at site B1s occurs, as 27SA3 is accumulated.

This comment may be based on a misunderstanding of the pre-rRNA processing pathway. Site B1S is not cleaved, it is generated by exonuclease digestion from site A3. The data shown are consistent with previous publications.

Due to the overexposure it is really hard to tell, but it does seem that there is no reduction in the strong DMS independent stop at B1S in the Cic1 strain, which suggests that perhaps the authors are not using RNA from Cic1 depleted strains.

This comment may be based on a misunderstanding of the data presented. As stated in the legend to Fig. S4 the primer extensions shown in Fig. S4C are taken from the same gel as that shown for DMS modification in Fig. S7. Fig. S4 presents a shorter exposure precisely to allow the change at B1L/S to be seen. The Cic1 strain used was verified. We have replaced the gel shown in Fig. S7 with a shorter exposure. It is clear from this gel (Figure S7, lane 7) that there is far less processing to B1S in Cic1 depleted cells.

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Revised point: The authors have also carried out this experiment (Figure 7), and produce data of extraordinary quality, but this experiment has possibly even more flaws than the previous one, as described below.

It does not seem that the authors know that in the interpretation of the DMS gels, one needs to go up 1 nucleotide in the sequencing lane, as the RT falls of at the modification, but the sequencing stops 1 nucleotide after the ddNTP incorporation). To my eye it seems like most residues are misassigned by one nucleotide (it is very hard to tell for sure though because the figure is so small, perhaps showing a full page figure in the supplemental data would help; this comment also applies to Fig. S7). Minimally, the authors must label the entire gel better. Residues that they want to discuss should be labeled with the residue number, e.g. C2 and C5, on the gel. This would help navigate the gel and the corresponding secondary structure map in Figures 7C and D. Considering that every residue is shifted one nucleotide it also seems like many of the most obvious changes are actually Us (the most prominent stops in stem 2 and 3 are uridines). DMS modifies A and C, as well as G. However, to detect G modification one needs to carry out aniline cleavage, which was not done. "U"s cannot be modified by DMS unless there is a tautomeric shift, which requires a pKa change. Thus, "U"s should not be interpreted. This will remove many of the interesting nucleotides from the authors' analysis. Had they labeled the gel better with residue names and numbers, this would have been immediately obvious.

The suggestion that the major sites of DMS-induced primer extension stops correspond to U residues (which are not susceptible to DMS modification) seems quite surprising. As the referee notes we have previously published in vivo DMS modification data and are aware of where reverse

transcriptase stops. We have analyzed the samples from independent experiments on many different gels using various oligonucleotides and used that information to highlight the modifications in the secondary structure. The scans presented in the manuscript (and supplementary Figure S7) are of high quality; all have the exact same loading order and clearly highlight the changes in RNA secondary structure/DMS reactivity found in ITS2 in a single panel. We acknowledge that in some cases the sequencing ladder does not line up perfectly with the samples and from our experience we find that this is often due to electrophoresis conditions (smiling, temperature of the gel, etc) and in part the presence residual salt present in the primer extension reactions which can cause a shifted band pattern. Especially in the center of the gels and the top do we often see primer extension stops between two adjacent nucleotides of the sequencing ladder.

This is not an uncommon technical problem. For example, similar band shifting can be seen in the 2011 Lamana and Karbstein paper (Figure 4A). This band shifting is apparent mostly in the top of some of our gels (for example compare lanes 7-9 in the top of the gel) where the bands are more compressed.

To overcome this potential problem we used gels from previous control experiments to precisely determine the stops near the 5' end of ITS2, including the most important two nucleotides C2 and C5. These data, now included as Supplementary Figure 8, also allowed us to more accurately map some of the differences in DMS modifications between *in vivo* and *in vitro* modified RNA in the 5.8S rRNA. We loaded primer extension reaction from different oligonucleotides on the same gel and it can clearly be seen that the ladder and primer extension stops do not perfectly align at the tops of the gels, particularly for the C2 and C5 modification, but align well on longer migration. We have also labeled the nucleotides in the gel, as suggested by the referee. We believe that this makes the interpretation of the results easier. We also now show a larger scan in Figure S7.

Finally, we also reanalyzed all of the chemical modification data to make sure absolutely sure that we correctly interpreted the data. Of all the stops analyzed, only two appeared ambiguous (C35 and C37), and we eventually decided to label one of these stops as a U (indicated with an asterisk) and the other as an A (A34). These changes have been included in the revised Figure 7.

Furthermore, labeling on the secondary structure does not correspond to what I can see in the gel. Minimally, there is no data shown for the modifications labeled as such in stem VI (Fig. 7C,D),

The gel showing this region was omitted simply for reasons of space. However, we have now included this in the revised Figure 7. This region is close to the primer and the DNA sequence (which is weakest near the primer) is not readily visible at the exposure needed to see the primer extension (which is strongest near the primer). However, the exact positions of these three nucleotides were mapped on longer exposures and in an additional experiment in which manganese was added to the sequencing reaction to give stronger signals close to the primer.

the number of strong stops on the secondary structure does not even correspond to the number of strong stops in the wild type strain, some modifications are not mapped etc..

Stops that were also found in unmodified samples or were clearly at U's were not all highlighted in the secondary structure, as these were considered irrelevant. Furthermore, we only highlighted nucleotides on the secondary structure that we could confidently map. For example, we could not unquestionably map the stops between A2 and A3 (sup Fig 7) and therefore decided not to include these in the secondary structure. Again, we would like to emphasize that the goal of this study was not to do a comprehensive secondary structure of the 27S pre-rRNAs. This would go well beyond the scope of the current manuscript. We would like to refer the reviewer to the Lamana and Karbstein paper in JMB, which discusses ITS1 folding.

The secondary structure is also messed up in panel D at stems 1 and 2. This reflects sloppiness of the authors, the same kind of sloppiness that led to the mix-up of strains before.

The EMBO Journal did not allow us to upload a PDF of the figure at resubmission, but instead generated the PDF automatically from our Illustrator files. This formatting changed the font in all the main figures and this was only discovered after submission. This is indeed very annoying, and

we apologize for the inconvenience. The supplementary figures, however, were not reformatted and these are of high quality. We have hopefully corrected this problem for the second resubmission.

The data from the depletion strains fail to support the CRAC data. For example, the analyzed sites overlap with the suggested binding site for Cic1 and Nop15, based on CRAC analysis. However, depleting these proteins does not produce enhanced modification at that site, which would be a very strong prediction from direct protein binding.

We disagree with this conclusion. We clearly see an increase in modification in the Nop15 binding region when the protein is depleted, most notably in stem III (IIIA in the ring model in Figure 7) and directly adjacent to this region (i.e. nucleotides A34, C49 and A54). One could interpret this as an RNA structural rearrangement but because these modifications are not observed *in* vitro and the CRAC data show that Nop15 binds in that region, we concluded that Nop15 was protecting that region from DMS modification *in vivo*. Indeed, in the case of Cic1 we do not see increased reactivity at the major cross-linking site in ITS2. A plausible explanation for this is that the nucleotides in the cross-linking site that could be modified (G25, A24) by DMS are likely protected by Cic1 in the parental strain but base-paired in the absence of Cic1 and therefore escaped detection.

The same has been previously found for Nob1, where in vivo DMS showed protections different from those observed by CRAC.

We are unclear what point the referee wishes to make. The site that we analyzed by DMS modification was the region surrounding the site D cleavage not the Nob1 binding site. The reviewer should realize that CRAC is not exhaustive (see comments below) since it is biased towards protein-pyrimidine contacts. We pointed this out in our previous paper that lack of cross-linking does not mean that a protein does not interact with RNA, and that was one of the reasons we performed chemical foot-printing on the D site region.

While the statistics of this are certainly weak (only three analyzed strains), they do suggests that CRAC does NOT report on direct binding as asserted by the authors, but likely on nearby binding, consistent with findings on Prp43 crosslinking by the authors. While I do not believe that this has any serious impact on the usefulness of the technique or even the interpretations herein, I do think that this is an important point, which must be discussed, as it is currently believed in the community to do so.

We disagree with this comment: CRAC and related CLIP techniques do report sites of direct RNA-protein binding. We would refer the referee to our work on the binding sites for Nab3 and Nrd1 (Wlotzka et al., EMBO J. 2011), to the review by Licatalosi and Darnell (Nat. Rev. Genet. 2010) and to the many other recent papers that have made use of RNA-protein cross-linking. With respect to Prp43, the referee's interpretation of the data is quite different from ours, and from that of the referees of that paper. We reported several major and minor binding sites, but we believe that all of these represent sites of direct protein-RNA interaction. It is possible that referee may have in mind a distinction between "binding" by RNA sequence/structure recognition and other "contacts". In the case of r-proteins (for which the crystal structure is available) the "binding site" can be comprised of multiple RNA segments that are dispersed in the primary sequence. We would contend that these all represent direct RNA-binding sites, even though they may not all reflect sites of sequence-specific RNA recognition.

I would even encourage the authors to try to figure out what the radius is for CRAC modification, or if there are any constrains (i.e. only modifications in the minor groove, as found for Fe-EDTA mapping), to help future interpretations of the technique. I must stress though that such an analysis would go clearly beyond the current manuscript and is only for the authors' future considerations.

This comment may be based on a misunderstanding of the technique. UV is a zero-length cross-linker and there is no radius for modification. The RNA and protein must be in direct contact for cross-linking to occur.

Finally, the experiment was carried out to provide evidence for conformational switches promoted by the A3 cluster proteins. There are exactly 2 nucleotides that differ in these strains, C2 and C5. While the changes in their modification are consistent with the model of the ring and hairpin switch

and a role of Cic1 and Nop15 in either promoting this switch, none of the other predicted changes can be seen; thus, the data simply seem to suggest that Cic1 and Nop15 prevent the formation of a longer stem II.

We did not expect to see all the predicted changes, as Cic1 and Nop15 are only two of the many proteins involved in ITS2 processing. However, the data strongly suggest a role for Cic1 and Nop15 in maintaining a more flexible structure and predict that these proteins are directly involved in the proposed RNA conformational switch. The accuracy with Peculis and Greer predicted these binding sites seems remarkable. We have, however, softened our statement about the role of Cic1 and Nop15 in ITS2 folding on page 11 in the main text: "We conclude that Cic1 and Nop15 binding is necessary to maintain or establish a more flexible and open structure in the 5' end of ITS2, potentially preventing premature formation of the hairpin structure".

I am also not sure that I would have done the experiment this way. As pointed out by the authors, the DMS reports on all 27S RNAs, in the wild type strain that is mostly 27Sb, and some 27SA2, hardly any 27SA3, which the authors want to analyze; it seems the right strain for analyzing 27SA3 would be one deficient for A2 cleavage, so that 27SA3 accumulates, perhaps even combined with mutations in Rat1 to stall processing after A3. This strain then I would have analyzed +/- Nop15, Cic1 etc.

This comment may be based on a misunderstanding of the pre-rRNA processing pathway. It is unclear which host strain the referee is recommending we use, since inhibition of A2 cleavage does not generally lead to the inhibition of processing from A3. The referee is perhaps confusing the 27SA3 pre-rRNA with the 23S RNA (the 5' product of A3 cleavage), which does accumulate on A2 inhibition. Moreover, the suggestion that we should analyze the effects of the A3 cluster mutants on the ITS1 structure in the background of a strain expressing three different, conditional-lethal mutations does not appear feasible.

3. Original point: Surprisingly, the Northerns in Fig. S4 do not actually show the phenotype of 27SA3 accumulation for Nop7, even though it has been published twice, including by the Tollervey lab. Furthermore, the phenotype from Nop7 depletion is different from the phenotype observed with Nop15 depletion, which is in line with the previously published Nop7 data. How could this be if depletion of one proteins leads to depletion of all proteins, as stated multiple times, so that Nop7 depletion should be identical to Nop15 depletion. Please clear up this discrepancy with previously published data and between data herein.

Revised point: This point has been sufficiently addressed. However, I noticed that other experiments previously labeled as Nop7 have been relabeled as Nop4. Has that been checked in every instance, or was it simply assumed that the mix-up happened every time.

The same strain was used throughout.

4. Original point: This model also explains the changes in Rat1 crosslinking upon depletion of Nop7 or Nop15; previous data has shown that upon deletion of the A3 cluster proteins the 27SA3 intermediate accumulates at the expense of the 27SBS; the simplest interpretation of these data is not that Rat1 recruitment is affected but that the crosslinks at the 3'-end of 5.8S and in ITS2 that are reduced upon Nop7/15 depletion occur in 27SB (the wild type most abundant species); in contrast the crosslinks at the 5'-end of 25S (which is base paired to the 3'-end of 5.8S) increase, similar to the levels of 27SA3, indicating that these might actually occur in the 27SA3 species, the relevant one; this is near the sites for Nop7 and Erb1; crosslinks e g in 5'-ETS do not change as the abundance of the intermediate in which these crosslinks occur does not change

Revised point: This point has been partly addressed. However, I do not understand the author's logic that Nop4 inhibits processing prior to A3 cleavage. That would mean A2 cleavage, and would suggest an effect on 18S production, which is NOT observed.

The primer extension analyses indicated a modest drop in A2 cleavage efficiency and this is consistent with previously published data (Berges et al 1994 EMBO J) showing that Nop4/Nop77 depletion delays A2 processing, leading to an under-accumulation of 20S and 27SA2 pre-rRNAs.

Furthermore, there is not a single reported strain, in which A3 cleavage does not occur but the ratio of 27Sb/27Sl is unchanged. In fact that is a hallmark of missing A3 cleavage, observed in the RNase MRP mutants.

This comment may be based on a misunderstanding of the pre-rRNA processing pathway. 5.8SL:S imbalance is seen is strains that are inhibited only for cleavage at A3 (such as MRP mutants) or subsequent exonuclease digestion (such as Rat1 mutants). However, several mutant strains apparently inhibit processing at both A3 and B1L, leading to loss of 27SBL and 27SBS without a clear imbalance.

Finally, the Northern analysis demonstrates that NO 27S RNA can be found. This suggests that in the absence of Nop4 27S pre-rRNA is rapidly degraded, consistent with the author's proposal that Nop4 helps bring together domains II and III of 25S rRNA. I suggest that Nop4 stalls after A3 cleavage, hence no effect on the ratio of 27Sl/SB, but that the accumulated intermediate (27SA3, or 27SB) is rapidly degraded. The remaining RNA is wild type, consistent with the observation that there are no differences between the RNA from this strain and the wild type strain by primer extension/ DMS.

This is an interesting hypothesis. We believe that the DMS probing experiments revealed parts of the secondary structure of the 35S pre-rRNA, since this accumulates to high levels in Nop4 depleted strains.

If the authors want to make a point that Rat1 crosslinking depends on a specific protein, they need to look at the same RNA species +/- the desired protein, i.e. Rat1 mutants that accumulate 27SA3 in the presence and absence of their favorite protein. If they don't want to include this experiment, which requires strain building and new experiments and is difficult, they should not draw this conclusion, but say that in model a) Rat1 recruitment depends on these proteins, or that b) it simply reflects the different abundances...and that further experiments must be done to differentiate between these two.

These comments do not appear to reflect the changes made in the revised text. We have, as requested, removed the claim that specific Rat1 binding is induced by specific proteins.

5. Why are the sites for the other proteins not included in Figure 4, instead all of these Rpl are shown? In addition, the lesser sites are also not shown, but likely relevant (H10~H66, for Nop12), why omit data selectively?

Revised point: H8 for Nop12 is still not shown.

We have added this to Figure 4.

That data is also intriguing, as it shows crosslinking to only one side of the presumed duplex between 5.8S and 25S rRNA. While one could say it binds to one site of the duplex, the labeled sites of interaction are over more than one turn of the duplex, which means both strands must contact that one side, making these results surprising.

These are the results obtained. We are unclear what point the referee wishes us to address.

I feel that the data herein map the binding sites for the A3 cluster proteins near 5.8S rRNA, and near the binding sites for the RpL depleted in the absence of the A3 cluster proteins (is that reference submitted from the Tollervey lab?, if so, it should be included in this manuscript).

The work on the ribosomal proteins was performed in the lab of John Woolford, with whom we have been communicating our results. Their MS is currently in revision in EMBO journal and their data fit very well with the work described in our manuscript.

They also show that CRAC crosslinking does NOT report on direct sites of interaction, as previously suggested.

We disagree, see comments above.

Furthermore, they provide data localizing Rat1 near its processing sites. Depletion of Cic1 and Nop15 and in vivo DMS probing indicates that binding of these proteins could prevent formation of a longer stem II in ITS2, but provides no convincing evidence for a role of these proteins in the proposed switch between the hairpin and ring models.

Because it is currently not possible to assemble yeast pre-60S complexes in vitro (or even ribosomal subunits) or obtain homogeneous populations of pre-60S particles and because we lack structural information on these particles, it will be very difficult to provide direct evidence for Cic1 and Nop15 involvement in the conformational switch. We are, however, in the process of developing new tools that will allow us to exactly address these questions.

The conclusion that there's a switch in ITS1 awaits further analysis (Fig.S7).

As stated above, the goal of this study was to obtain more detailed insights into the role of a subset of ribosome assembly factors in pre-rRNA processing, not to study a conformational switch in ITS1. Work on this particular subject has recently been published by the Karbstein lab in JMB, to which we refer the reviewer.

The interpretation that Rat1 binding requires A3 cluster proteins is also remains unsubstantiated.

As mentioned above, we have, as requested, removed the claim that specific Rat1 binding is induced by specific proteins.

If the authors were to substantially revise the manuscript to reflect these pared down conclusions, I would think it is appropriate for publication, although in my opinion it is not clear that that should be in EMBO, as functional data are entirely lacking.
I will however leave that for the editor's judgment.

Referee #3

This reviewer thinks that the revised version of this manuscript has significantly gained quality regarding the original one.

The authors have practically satisfactorily answered to this reviewer's comments. Most points have adequately been explained. Moreover, most of the additional experiments I suggested have been done and they are convincing. I also believe that points raised by the other referees have properly been addressed.

I still find some critique although minor; I encourage the authors to make the effort to address it:

Point (a) has not been corrected yet, thus sentence starting by "The 5.8S 5' end is generated by Rat1" has not been modified yet to "5.8Ss".

We have altered this to read “The major 5.8S 5' end is…..” The problem was that the term 5.8Ss will be unfamiliar to many readers, and we did not want to use the abstract to introduce the definitions of $5.8S_S$ and $5.8S_L$ as we felt this would be distracting.

Point (e). Figure S4 is still not totally satisfactory. Authors nicely show accumulation of 27SA3 upon depletion of NSA3, ERB1, NOP7, NOP15 and deletion of NOP12. Unfortunately, the primer extension shown in Fig S4C has no enough resolution to distinguish between 27SBL and 27SBs pre-rRNAs.

We now show an enlarged view of the B1L/S region in the revised version of Fig. S4, which allows the sites to be better visualized. The effects of depletion of these proteins on processing at sites B1L and B1S have all been previously published, and the data presented are consistent with the literature.

Point (h). The authors claimed that drawing the numerous Rat1 binding sites would create a very busy figure which would not be very informative, but honestly, Table S2 provides a never ending list of raw data (even after Novoalign cooking !) of very difficult digestion.

We included Table S2 in at attempt to address the initial comments of the referee. We felt that the table had the advantage of allowing the interested reader to assess the relative numbers of hits at different locations. We could not see an easy way to present this information on a cartoon showing all Rat1 hits superimposed on the secondary or 3D structures. As can be seen from the table, substantial regions of the pre-rRNA are covered by Rat1 cross-linking, but the distribution is far from even. Simply highlighting these entire regions seemed to us less informative than the data as presented in Table S2.

I wonder if a Figure on the pre-rRNA showing only that Rat1 CRAC related sequences where frequently mutations were found (i.e. boxes as in the case of Figure S3) could be more useful.

We have included the map of all mutations on a cartoon of the spacer regions in a new Figure S9.

Two additional typos:
Figure 5B. Please label first panel on the left with 27SA/B (I guess).

Done

Figure 5C is (similarly to previous point e) still no perfect since levels of 27SBL and 27SBs could not be interpreted from such an overexposed film.

We have inserted an additional panel showing a shorter exposure in the revised Fig. 5.

Legend to Figure S4. Please correct sentence "scan shown in Figure S6" to "S7".

Done

3rd Editorial Decision 24 June 2011

Thank you for sending us your re-revised manuscript and your detailed point-by-point response. In the meantime I have consulted with referee 3 on this final version and your response to referee 1. He/she is very supportive, and taking into account all points put forward, I have now come to the conclusion that the manuscript will now be publishable in The EMBO Journal.

Still, there are two editorial issues with the supplementary material that need further attention. First, the legend to figure S7 appears to miss information on panels B and C.

Second, prior to acceptance of every paper we perform a final check for figures containing lanes of gels that are assembled from cropped lanes. While cropping and pasting may be considered acceptable practices in some cases (please see Rossner and Yamada, JCB 166, 11-15, 2004) there needs to be a proper indication and explanation in all cases where such processing has been performed according to our editorial policies. Please note that it is our standard procedure when images appear like they have been pasted together without proper indication (like a white space or a black line between) and/or explanation in the figure legend to ask for the original scans. In the case of the present submission there is one panel that does not fully meet these requirements: S4C, bottom panel. I therefore like to kindly ask you to send us a new version of the manuscript that contains a suitably amended version of this figure as well as an explanation in the figure legend that all lanes come from the same gel. Please be reminded that according to our editorial policies we also need to see the original scans for the figure in question.

I am sorry to have to be insistent on this at this late stage. However, we feel that it is in your as well as in the interest of our readers to present high quality figures in the final version of the paper.

Thank you very much for your cooperation.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #3 (Remarks to the Author):

Authors have satisfactorily answered to this reviewer's comments

3rd Revision - authors' response 27 June 2011

We are resubmitting a revised MS with modifications to the legends to Figures S4 and S7 as requested, and a revised version of Fig. S4C. The original gel used contained a additional blank lane, which was removed simply to maintain the register with the other gels shown in the panel. This is now indicated by a line in the revised figure. The scan is included in the SI.

